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Interferon-Gamma (IFN-γ) as a Potential Radio- and Chemo-Protectant

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Interferons (IFN) have had increasing clinical usage in the treatment of a variety of disorders, at times being used in combination with chemo- or radiotherapy. However, interferons may have inhibitory effects on hematopoietic stem cell proliferation and the effects of this cytokine's use on long-term hematologic function have not been studied. We performed the competitive repopulation assay in the murine system, using cells exposed to irradiation or single-dose chemotherapy with or without concomitant IFN-γ use. IFN-γ alone had no deleterious effects on hematopoietic stem cell productivity. We measured the repopulating ability of exhaustible multilineage precursors that were present at early stages of marrow repopulation after competitive repopulation (30 days). These progenitors were minimally impacted by cyclophosphamide (CTX) with or without IFN-γ. Irradiation (XRT) and CTX alone produced significant repopulating defects in the most primitive hematopoietic stem cell, PHSC. Addition of IFN to either treatment regimen resulted in protection of PHSC, with improved repopulating ability, although the levels of donor marrow reached control levels only when CTX and IFN were used together. The results of multiple use of IFN with chemotherapy must be studied further, but IFN may offer hematologic radio- and chemoprotection, in addition to its antitumor properties in clinical protocols for treatment of cancers. Am. J. Hematol. 58:218–223, 1998.

Key words: interferon; hematopoietic stem cells; radioprotection; chemoprotection

INTRODUCTION

Interferons, potent inflammatory cytokines, are being used in the treatment of an increasing array of clinical disorders. Besides having a direct antiproliferative effect on human cancer cell lines [1], the interferons are known to increase the responsiveness of cellular immune mediators such as natural killer cells or cytotoxic lymphocytes to other cytokines, which stimulate specific antitumor immune responses, whether in an MHC-restricted fashion or not [2,3]. They also promote the activation and aggregation within tumor tissue of these cells [3]. Its modulation of extracellular matrix protein and restoration of normal adhesion molecule interactions has implications for the treatment of disorders such as chronic myelogenous leukemia [4,5].

These varied anti-tumor activities of interferons have led to the use of interferon-gamma (IFN-γ) in a number of clinical settings, and with widely disparate results. It has been used in combination with chemotherapeutic agents in the treatment of colon, small cell lung and metastatic renal cell carcinoma, high-risk cutaneous melanoma, as well as chronic myelogenous leukemia [6–12]. Because of the demonstrated potentiation of radiation's antiproliferative effects, it has also been given together with radiation in an effort to improve radiation's efficacy in other tumors [13,14]. After transplantation, it has proven useful in the generation of graft-vs.-host (and possibly graft-vs.-leukemia) reactions, which may be beneficial to individuals undergong autologous marrow transplantation [15–17].

While cytokines have had increasing utility in clinical settings [18–20], the combination of cytokines with chemotherapy may not be an entirely innocuous combination [21] since they may exacerbate an already present marrow reconstitutive defect that may have associated long-term clinical consequences such as residual cytopenias and marrow hypoplasia [22–28]. The long-term hematologic effects of interferon usage with chemotherapy

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or radiation, which may also be quite damaging to marrow hematopoietic stem cells, are not known. We employed the competitive repopulation assay [29] to ascertain whether interferon with chemotherapy or sublethal irradiation leads to impairment of the repopulating ability of the most primitive hematopoietic stem cell, which is responsible for life-long marrow stem cell renewal. The results of these experiments are reported.

MATERIALS AND METHODS

Mice

Congenic strains of mice, C57B16/J (B6) and B6-*Hbb*^{d}-*Gpi-1*^{a} (GPI) (Jackson Laboratory, Bar Harbor, ME), were used in all experiments. They were housed and fed under standard conditions. All the mice used were females, aged 8 to 12 weeks old.

B6 and competitor B6-*Hbb*^{d}-*Gpi-1*^{a} mice have different alleles at their *Hbb* and glucose phosphate isomerase loci. B6 mice are homozygous for the *Hbb*^{s} locus specifying $\alpha_2\beta_2{}^{s}$ (single) hemoglobin (Hb), the Hb migrating rapidly on hemoglobin electrophoresis. They also produce Gpi-1^{b} glucose phosphate isomerase isoenzyme. The B6 congenic strain are homozygous for the *Hbb*^{d} locus, having $\alpha_2\beta_2{}^{dmaj}$ and $\alpha_2\beta_2{}^{dmin}$ (diffuse) Hb, migrating at intermediate and slow speeds on electrophoresis. They produce Gpi-1^{a} isoenzyme, which again has a different migratory or electrophoretic pattern from the B6 isoenzyme. While the mice also differ at the H-1 locus, this difference has been shown to have no significant bearing on transplantation outcome when the two congenic strains are used [30].

Irradiation and Chemotherapy Experiments

Recombinant mouse interferon-gamma, obtained from Genzyme (Cambridge, MA), was used in these experiments. The cytokine had a specific activity of 1×10^7 U/mg and ≥95% purity. Mice, 8 to 12 weeks old, were injected with 1 μg IFN-γ intraperitoneally (i.p.) 24 hr before the administration of a single dose of 200 mg/kg cyclophosphamide intravenously (i.v.) or 500 cGy of irradiation using a ^{137}Cs-irradiator. Cyclophosphamide (CTX) (Sigma, St. Louis, MO), a commonly used antitumor agent, has been used extensively by us and has been shown to significantly impair PHSC repopulating ability with just a single dose of 200 mg/kg [20,21], reducing the stem cell pool available for marrow reconstitution. Marrow cell numbers are restored to control or pre-transplant levels by 4 weeks after treatment [20]. Mice were sacrificed after 4 weeks and the marrow from bilateral femurs and tibias was placed in a single-cell suspension in α-medium or DMEM (Gibco, Grand Island, NY), prior to use in competitive repopulation.

Competitive Repopulation

Bone marrow concentration was adjusted to allow 10^6 donor (B6) cells to be mixed with 10^6 competitor (GPI) cells. Equal aliquots of the mixtures were injected into B6 mice that had received 1,100 cGy. Blood was obtained by retroorbital puncture at 30 and 150 days and then separated by density gradient centrifugation to allow separation of lymphocytes for glucose phosphate isomerase electrophoresis. Densitometric analysis permitted determination of the exact percentages of donor marrow.

Measurement of donor cell percentage in recipients at 30 days permits a study of marrow precursors that give rise to both myeloid and lymphoid lineages, but that lack the long-term repopulating ability of PHSC [31]. Precursors measured at this early date are exhaustible, and probably contribute the most to early repopulation of marrow after the radiation-induced myeloblation for transplant, although some minor additional reconstitutive effort by PHSC at this early date cannot be ruled out entirely. These cells can be separated from PHSC on the basis of limiting dilution and surface antigenic characteristics, and are not identical to CFU-S [32,33]. The PHSC proliferative effort then becomes predominant after 30 days, with PHSC assuming the major reconstitutive role after this early date. Analysis of donor percentages at the later dates measures PHSC repopulating capacity.

Statistical Analyses

Precursor numbers can be referred to as relative repopulating units (RU) and can be estimated by using the following formula [34]:

Relative repopulating units (RU) = (%) (no. of 10^5 untreated competitor marrow cells)/(100-%), where % = percentage donor cell type.

One RU is defined as the repopulating ability of 10^5 untreated competitor marrow cells. If the donor cell population functions normally, 10^5 donor cells should contain approximately 1 RU and repopulate as well as 10^5 untreated competitor marrow cells. If, however, fewer RU are present, it suggests that the number of cells available among donor cells that are capable of marrow reconstitution are fewer than those found in a comparable pool of competitor cells, and repopulating abilities per cell are reduced or both.

Two to three replicates of each experiment were performed. Levels of significance for comparison of results were determined by ANOVA, with results expressed as means ± standard error of the means.

RESULTS

Measurement of exhaustible multilineage progenitors (EMP) repopulating ability was made 30 days after com-

TABLE I. Results of Competitive Repopulation After Interferon (IFN)-γ and Cyclophosphamide (CTX) or Irradiation at 30 Days*

Treatment groups	% Donor (B6) cells	Total BM cells	CRU/10^5 cells	Total CRU/donor	N
Control	52 ± 3	5.4×10^7	1.1	594	20
XRT	20 ± 3	4.6×10^7	0.3	138	16
CTX	44 ± 2	5.8×10^7	0.8	464	25
IFN	52 ± 1	4.7×10^7	1.1	517	6
IFN/CTX	44 ± 4	5.9×10^7	0.8	472	9
IFN/XRT	28 ± 4	4.9×10^7	0.4	196	11

*Data collected 30 days after marrow transplantation are shown. Control groups were untreated. C57B16/J (B6) mice received 500 R (XRT) or 200 mg/kg CTX as a single intravenous (i.v.) dose. IFN-γ was administered 24 hr before either treatment in a dose of 1 μg given i.p. For competitive repopulation, equal numbers, e.g., 10^6 cells, were used for the bone marrow mixture from competitor (GPI-1A) and donor (B6) animals, and standard aliquots of this mixture were given to lethally irradiated recipients (B6). Results are shown as mean ± standard error of the means (S.E.M.), while N = the number of mice used for each group. Donor marrow cell counts are shown after treatment; total repopulating units (RU) and concentration of RU are also given. Concentration of RU is represented per 10^5 untreated competitor cells.

petitive repopulation, and results are shown in Table I. The animals in the control group received aliquots of marrow containing equal proportions of donor and competitor cells. As expected, donor cells contributed approximately 50% of the marrow repopulating effort. Interferon alone did not impair EMP repopulating ability, with the single dose used. Radiation, as previously demonstrated, was deleterious to the repopulating ability of EMP; the donor percentage dropped to 20 ± 3%. In these experiments, EMP were minimally affected by a single dose of CTX at the dose employed of 200 mg/kg, with 44 ± 4% donor cells seen. Nor did a combination of interferon and CTX significantly damage repopulating ability; the addition of interferon before CTX administration did not lead to any exaggeration of the minimal drop in donor percentage from control levels (to 44 ± 2%) seen with CTX alone. The addition of interferon prior to irradiation led to an insignificant rise in donor percentage to 28 ± 4% from 20 ± 3% after that treatment modality alone.

Calculation of the concentration of repopulating units and total numbers per animal revealed an expected value of about 1 RU/10^5 competitor cells for control. The administration of XRT yielded a 2/3 reduction in RU concentration to 0.3, and a decrease in total RU numbers to 23% of control numbers, to 138 and 594. CTX administration resulted in reductions in RU numbers and concentration, but these numbers were approximately 80% of control values. Again, use of IFN-γ before CTX did not greatly alter these results. IFN-γ with XRT led to slight increases in RU, whether in concentration or absolute numbers, with total RU measuring 196; this can be compared to XRT alone, which again decreased RU numbers to 138. No treatment regimen resulted in significant declines in marrow cell numbers.

Effects on Long-Term Hematopoietic Progenitor Cells or PHSC

The long-term repopulating ability of PHSC was measured at 150 days after competitive repopulation. Results are shown in Table II. We have previously demonstrated severe impairment of PHSC repopulating ability after XRT and these results substantiate this effect. Repopulating ability dropped drastically, with the donor percentage being 4% after prior exposure to XRT. These results are statistically significant ($P < 0.001$). Administration of a single dose of CTX resulted in a decline of donor percentage from 50 ± 4% for the control group to 33 ± 3% ($P < 0.05$). With preadministration of interferon before CTX, donor percentages increased to control levels, with a value of 47 ± 4% being recorded, a significant improvement in PHSC repopulating ability ($P < 0.05$) when compared to CTX alone. IFN provided a beneficial effect when given prior to XRT, as well, although not completely protecting PHSC. Donor percentage was increased to 28 ± 7% from 4 ± 2% ($P < 0.05$). Interferon itself did not adversely affect PHSC repopulating ability. In fact, donor percentage rose to 65% with just a single dose of IFN-γ.

Exposure of marrow to XRT led to severe drops in RU numbers and concentration to 18 and 0.04/10^5 cells (from 540 and 1.0/10^5 cells, respectively, for control cells). The use of IFN-γ 24 hr before administration of XRT produced an at least 10-fold increase in these numbers, with RU concentration being 0.4/10^5 cells and the total numbers of RU increasing to 196. CTX use alone led to reductions in RU concentration and total numbers. RU numbers were 406/donor, compared again to 540 in control animals. The use of IFN-γ before CTX was restorative, since RU numbers then rose to control levels. IFN-γ alone resulted in increases in absolute numbers

TABLE II. Results of Competitive Repopulation Assay at Days 150 After Interferon (IFN)-γ and Irradiation or Cyclophosphamide (150 Days)†

Treatment groups	% Donor (B6) cells	Total BM cells	CRU/10^5 cells	Total CRU/donor	N
Control	50 ± 4	5.4×10^{10}	1.0	540	21
XRT	4 ± 2*	4.6×10^{7}	0.04	18	15
CTX	33 ± 3*	5.8×10^{7}	0.7	406	24
IFN	65 ± 3	4.7×10^{7}	1.9	893	7
IFN/CTX	47 ± 4	5.9×10^{7}	0.9	531	8
IFN/XRT	28 ± 7*	4.9×10^{7}	0.4	196	10

†Information regarding experimental groups and abbreviations is detailed in Table I. The results are from competitive repopulation experiments analyzed at 150 days. Results are given as means ± S.E.M., while N represents the number of mice in each group.
*Significant differences ($P < 0.01$–0.001) between the indicated group and the controls as determined by ANOVA.

and concentration of RU. Reductions (or increases) in RU number were not proportionate to declines in marrow cell numbers.

DISCUSSION

Interferon's antitumor and inflammatory characteristics have made it a valuable adjunct in treatment protocols for a variety of cancers. However, IFN-γ is an inhibitor of hematopoiesis and has also been implicated recently in the pathogenesis of aplastic anemia [35,36]. Transduction of the IFN-γ gene into human stromal cells resulted in secretion of IFN-γ and resultant inhibition of long-term culture-initiating cell function, in addition to that of more committed progenitors. Whether the long-term hematologic impairment is the result of induced stem or progenitor cell apoptosis or of direct proliferative cell inhibition is not entirely clear, but IFN-γ is responsible for the induction of nitric oxide synthase, which inhibits hematopoiesis in a dose-dependent fashion [37]. The activation of fas antigen by IFN-γ also may potentiate hematopoietic suppression [38].

While the effects of IFN-γ and chemotherapy on the long-term repopulating abilities of hematopoietic stem cells have not been studied previously, hematologic toxicity is a recognized side-effect of many of the regimens utilizing interferons with other anti-tumor agents [7,10,39]. Gallichio and his associates reported increased hematopoietic toxicity with combined IFN-γ and dideoxynucleoside therapy, utilizing in vitro assays [40], although parallel experience has not been noted for IFN-γ with chemotherapy. The ability of interferons to potentiate the toxicity of irradiation in cell lines or tumor tissue is well known [13,14] but again the hematopoietic system has not been studied.

In addition, the full extent of any cytokine's impact on long-term hematopoietic function is not yet known. Some cytokines such as interleukin-1 (IL-1) and stem cell factor (SCF) have been reported to have radioprotective and/or chemoprotective properties [41–43]. These conclusions have been based on survival data and in vitro assays. In a manner similar to that used with IFN-γ, IL-1 resulted in a 75–90% survival rate in mice after a treatment that otherwise would be 100% fatal [42]. The mechanism of such protection is not entirely clear. SCF has been shown to increase the number of progenitor cells in mice [44–46], while IL-1 may increase aldehyde dehydrogenase content in stem and progenitor cells, promoting resistance to the toxic effects of some cytotoxic agents, such as cyclophosphamide [47].

The concomitant use of cytokines with chemotherapy possibly increases the damage already induced by the cytotoxic drugs, especially since the clinical doses of cytokines are far in excess of their usual physiologic concentrations. This deleterious effect has been demonstrated by Hornung and Longo [25]. In performing murine serial transplantation after exposure of cells to multiple cycles of cyclophosphamide and GM- or G-CSF, they observed significant reductions in the restorative capacity of bone marrow after two to three serial passages when either cytokine was given after chemotherapy administration.

We had fully expected that IFN-γ, given in conjunction with CTX or irradiation, would further exacerbate the previously documented hematopoietic defect seen after these modalities [22–28]. In these experiments, a single dose of CTX and 500 R of XRT were sufficient to cause significant drops in PHSC repopulating ability, although EMP functional impairment was evident only after XRT. Consequently, IFN-γ exerted no or minimal beneficial effects on EMP function, but it did have demonstrable chemoprotective properties for and offered some radioprotection to PHSC, at least with this limited single-dose usage. The addition of IFN-γ after CTX almost completely reversed the hematopoietic defect induced by the chemotherapeutic agent, while repopulating ability was improved 7-fold after XRT.

This ability to protect cells from the deleterious effects of chemotherapy may stem partially from its inhibition of cell cycle progression or of initiation of proliferation of hematopoietic stem cell populations [35,36]. The prevention of cell cycling has been previously associated with stem cell-sparing after chemotherapy, albeit the noncycling populations have been more committed stem cell populations, such as CFU-S [48,49]. We speculate that IFN prevented exhaustion among the PHSC pool too by maintaining them in a quiescent state when chemotherapy was administered, thus making them less vulnerable to the drug's cytotoxicity. Alternatively, prevention of cycling among more mature progenitors may allow the PHSC pool to reserve its energies, and preserve its num-

bers for self-renewal rather than inducing them to cycle to replenish the numbers of committed precursors.

Selleri et al. [36] have postulated that chronic exposure of hematopoietic stem cells to IFN-γ and other inflammatory cytokines results in the induction of apoptosis and depletion of hematopoietic stem cell pools, a depletion that may be permanent and ultimately result in bone marrow failure. It is conceivable that XRT, more so than single-dose chemotherapy, might induce stromal damage with resultant inflammatory cytokine production, leading to exposure of hematopoietic progenitors to higher and more sustained or continuous levels of IFN-γ. Exacerbation of XRT-induced hematopoietic defect might result from IFN use because such extensive damage could be more difficult to repair.

We have used single-dose IFN-γ to avoid the possible effects of high and repeated exposure of PHSC to the cytokine, and to demonstrate the effects of limited use of IFN-γ with chemotherapy. Multiple doses of IFN with chemotherapy must be tested before any definitive statement about long-term implications of its combined use with chemotherapy can be made. Experiments are being considered to study this question further.

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